Ascorbic Acid and the Eye with Special Reference to the Lens

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The concentration of ascorbic acid is substantially high in various ocular tissues. As TABLE 1 indicates, the cornea and the lens are next only to adrenals and liver in this regard. The aqueous and vitreous humors of the eye are also substantially rich in their ascorbate content. The level of this substance in these two intraocular fluids is the highest among all the extracellular fluids of the body. It has previously been shown that the high concentration of ascorbic acid in the aqueous humor is due to an active transport of the nutrient by the ciliary epithelium across the blood aqueous barrier.[1] The aqueous humor can then perhaps act as a source of ascorbate to all the other ocular fluids and tissues including the lens, cornea, vitreous humor, and retina. The various ocular tissues are thus well fortified with this nutrient. Why nature has developed this mechanism of maintaining a high concentration of ascorbate in the aqueous humor, lens, and the other ocular tissues is as yet an unsolved mystery. Since ascorbic acid is a relatively strong reducing agent, it is quite possible that the role of this nutrient in such high concentrations may be to protect the tissues of the eye against the deleterious effects of the photochemical or the ambient oxidation reactions involving oxygen and its radicals. Previous studies have shown that exposure of animals to hyperbaric oxygen leads to the development of cataracts.[2] This has been found to be true in the case of humans also.[3] There now exists a substantial body of literature to suggest that the development of several oxygen-induced pathologic manifestations is initiated by conversion of the relatively inactive dioxygen to the superoxide radical anion and its consequent derivatization to other active species of oxygen.[4] The formation of the superoxide is considered imperative in many of the autoxidative and enzymatic reactions. An indirect proof of the concept that superoxide generation is toxic to the tissues is derived from the fact that most tissues are endowed with superoxide dismutase, an enzyme meant to effectively remove this free radical at a rate faster than would happen in its absence.[4] Thus the first task before us was to examine if the lens has superoxide dismutase (SOD). As summarized in FIGURE 1, that was found to be true.[5] The formation of adrenochrome from epinephrine, an O_2^--dependent reaction,[6] is inhibited by the dialyzed extracts of the rat lens, the inhibition being proportionate to the protein concentration. Similar reports on the presence of SOD in the lens were made simultaneously by other laboratories.[7,8] That the inhibition of adrenochrome formation by the lens extract is indeed specific to superoxide dismutase was subsequently proven by us following electrophoretic separation of the lens proteins on polyacrylamide gel and negative staining of the gel[9] for superoxide dismutase per se (FIG. 2).[5] Definitive evidence on the presence of SOD led us to postulate that superoxide generation (FIG. 3) in the aqueous humor might be a factor in the pathogenesis of senile cataracts and that ascorbate may be an element

TABLE 1. Concentration of Ascorbic Acid in Various Tissues and Fluids

	Man	Ox	Rabbit	Rat
Cornea	—	310	550	100
Aqueous humor	160	190	346	0
Lens	190	360	130	0
Vitreous	360	80	70	14
Ciliary body	—	87	196	
Retina		98	425	
Choroid		49	177	
Liver	70	290	170	240
Pancreas	11	125		
Adrenals	4000	1285	340	
Brain		170		
Cardiac muscle	—	38	—	—
Kidney	130	130	—	150
Saliva	0			
Seminal vesicle secretion	50	140		40
Urine	25	0		
Plasma	10	4.5	4.1	
GSF	12			
Leukocytes	165			

Values are expressed as mg/kg wet weight of the tissue or as mg/l of the fluid. The data have been compiled from those given in *Biochemists' Handbook*, D. Van Nostrand Company, Inc., New York (1961).

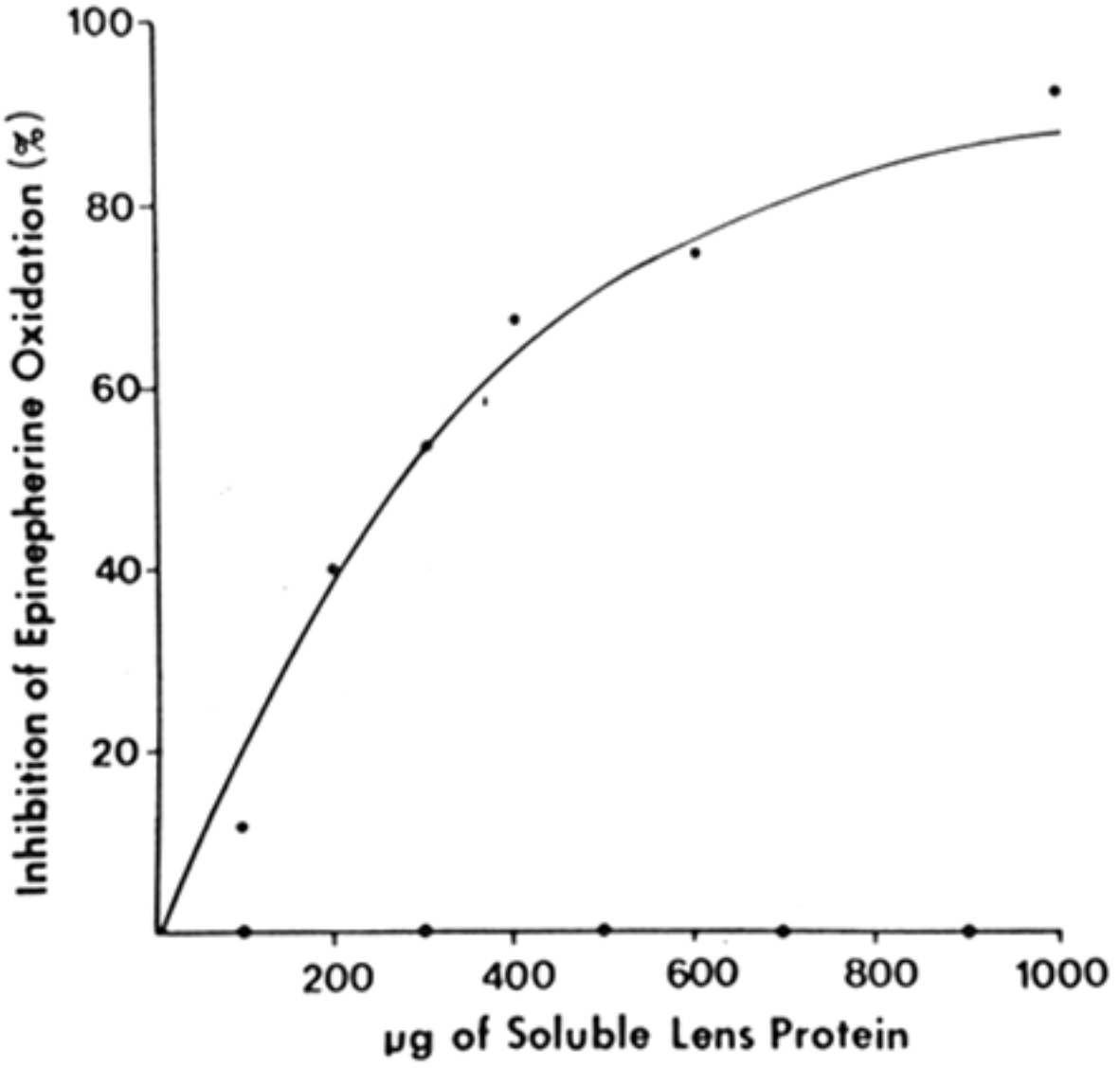

FIGURE 1. Inhibition of O_2^- dependent oxidation of epinephrine by dialyzed extract of rat lens homogenates. The rate of epinephrine oxidation was monitored by determining ΔOD_{460} nm. The reaction mixture consisted of sodium carbonate 0.05 M, pH 10.2, and epinephrine, 3×10^{-4} M. The reation was conducted in the presence and absence of the lens extract so that the values are expressed as the percentage of epinephrine oxidized in the absence and presence of the lens extract. The reaction was followed for a period of 3 minutes.[5]

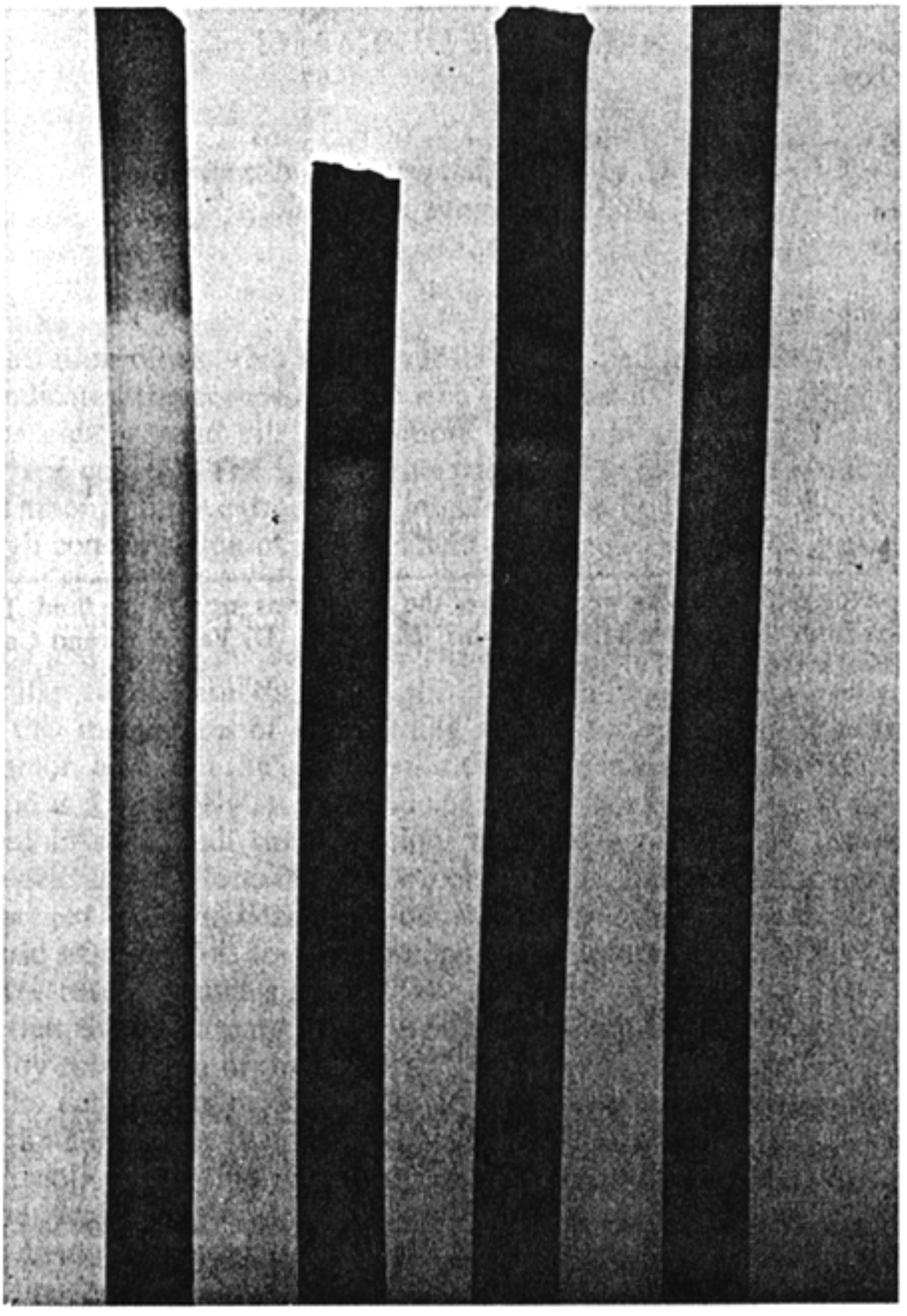

FIGURE 2. Identification of superoxide dismutase activity in lens. Soluble fraction of the lens homogenates representing about 200 μg of protein was electrophoresed on a 10% polyacrylamide gel and SOD activity, represented here by achromatic bands, was localized by negative staining according to the procedure of Beauchamp and Fridovich.[9]

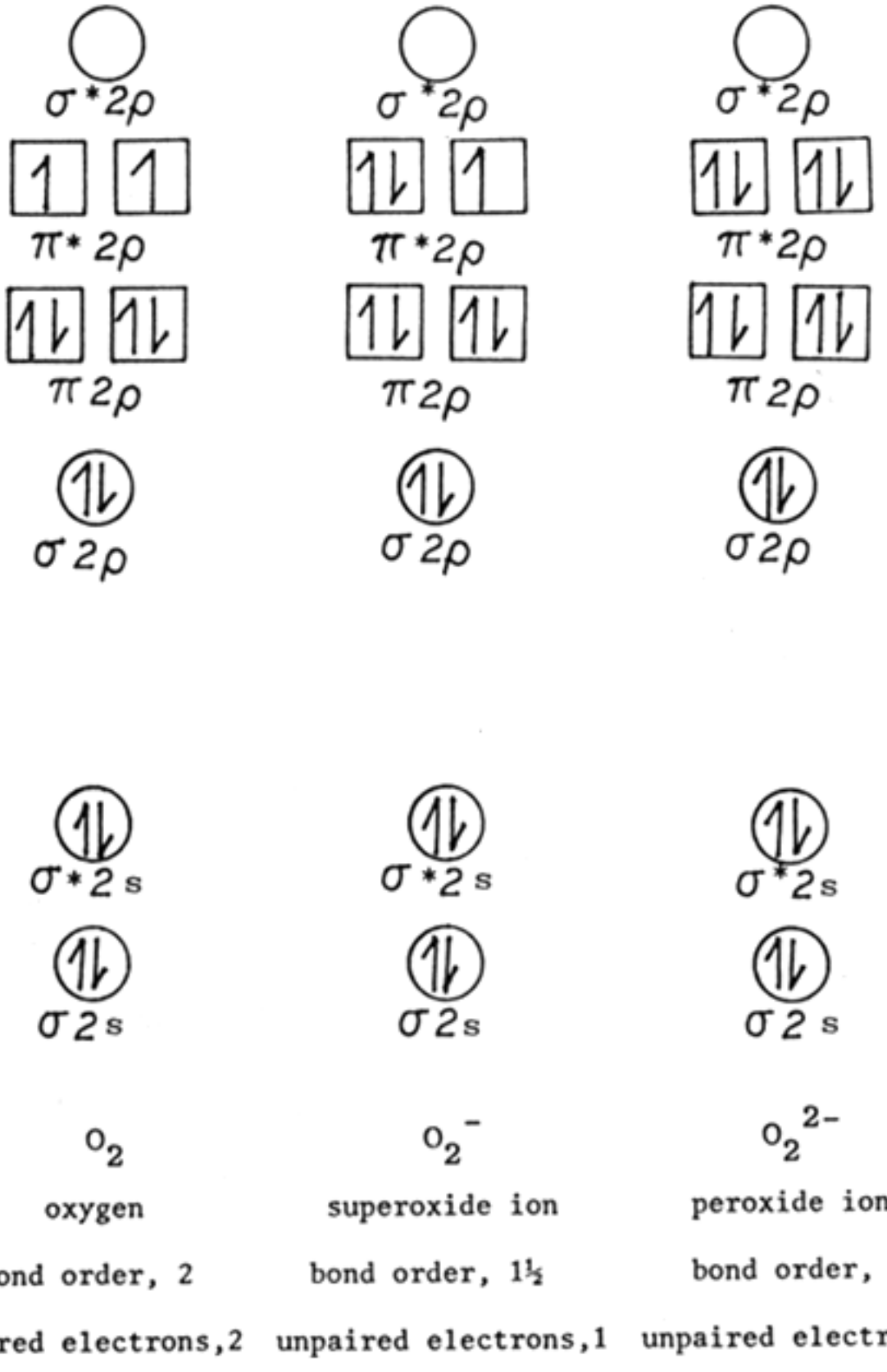

FIGURE 3. Electronic structure of oxygen, superoxide anion, and the peroxide anion. The orbitals have been indicated according to standard chemical textbooks. Electrons only of the principal quantum $n = 2$ have been taken into consideration.

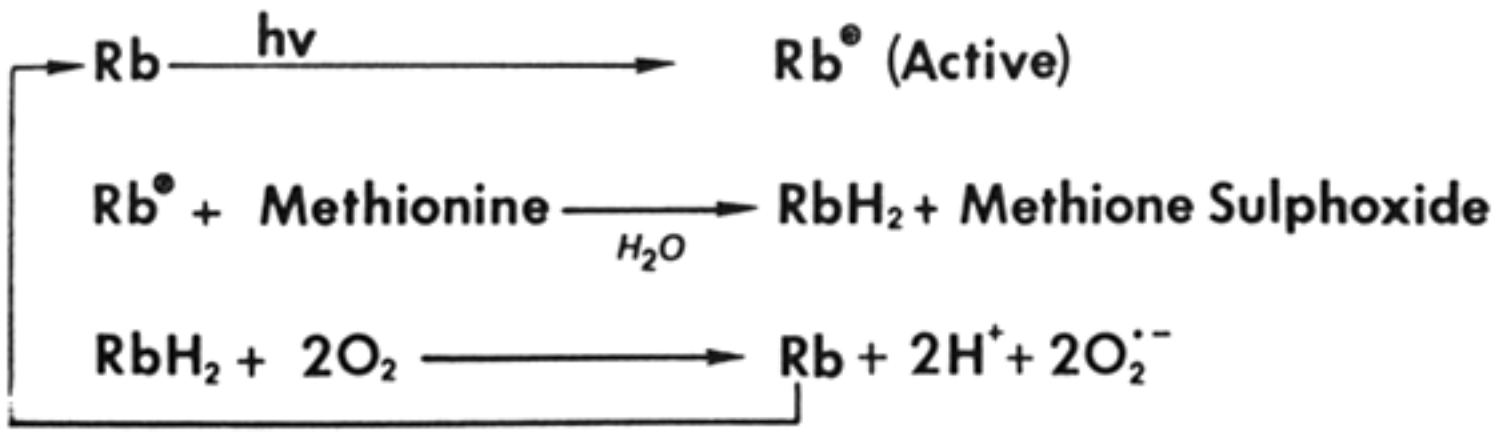

FIGURE 4. Photochemical generation of O_2^-: *Rb* = riboflavin, *hv* = light. Methionine can be replaced by other electron donors.

acting to thwart this pathological process by protecting the cell membranes against the toxic effects of O_2^-, hydrogen peroxide, and other oxygen derivatives.[5]

Several studies with bacterial and animal tissues have demonstrated that O_2^- is an important agent of oxygen toxicity.[4] We thought that this should be more so in the case of the ocular tissues. By virtue of the transparency of the cornea, aqueous humor, lens, vitreous, and retina, the intraocular chamber is constantly filled with light, at least during the long periods of the photopic vision. This provides a unique situation for an incessant photochemical generation of O_2^- and its derivatives in the vicinity of the tissue membrane sites, and makes oxidation of their components an imminent possibility. Excessive light, in fact, has long been implicated in the genesis of senile cataracts. The incidence of cataracts is higher in the geographic areas of the world that have a greater amount of sunlight. Thus, we developed a theory that light and oxygen may be acting synergistically in contributing to the overall cataractous process; the synergic action involves *in situ* photochemical generation of O_2^- in the aqueous and vitreous humors and its derivatization to other active oxidants.[10,11] The function of ascorbate may then be to attenuate this cataractogenic process.[5] An example of a reaction that may yield O_2^- and its derivatives continuously under the influence of light, as long as the system remains aerobic and the reducing equivalents are available, has been shown in FIGURE 4, the detailed reaction being described in FIGURE 5.

Experiments were, therefore, designed to test the hypothesis that such a photocatalytic system, like the one described in FIG. 4, would damage the lens physiologically. Such a test has been done by *in vitro* organ culture techniques. Briefly, freshly isolated rat lenses were cultured in riboflavin-containing medium in the absence and presence of the fluorescent daylight and their ability to actively transport rubidium against an electrochemical gradient was measured (FIG. 6). The results have been described as the distribution ratio of the ion between lens water and the medium

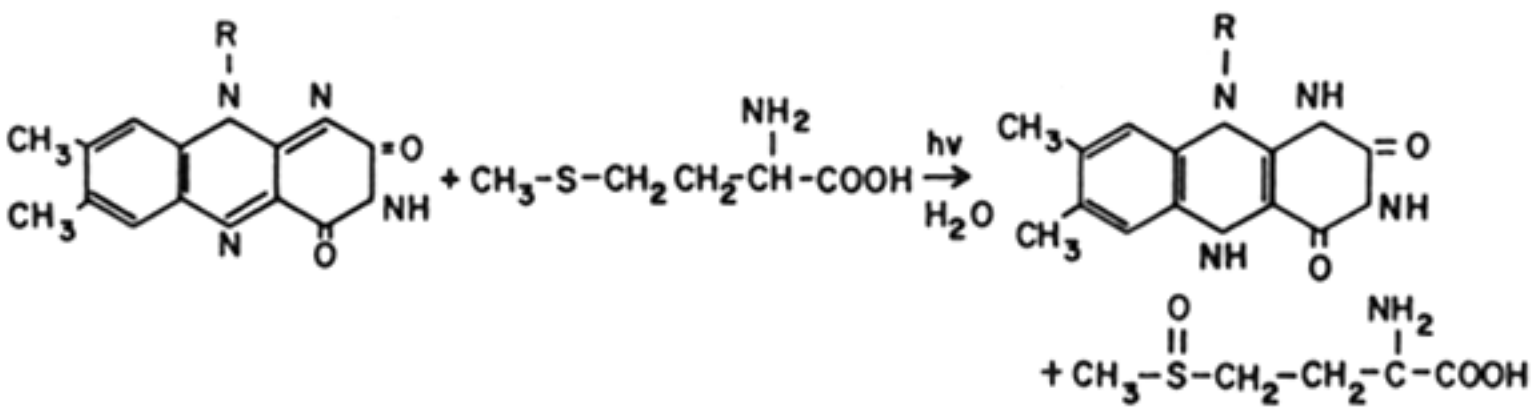

FIGURE 5. Reduction of riboflavin by methionine. The reaction is photocatalyzed.

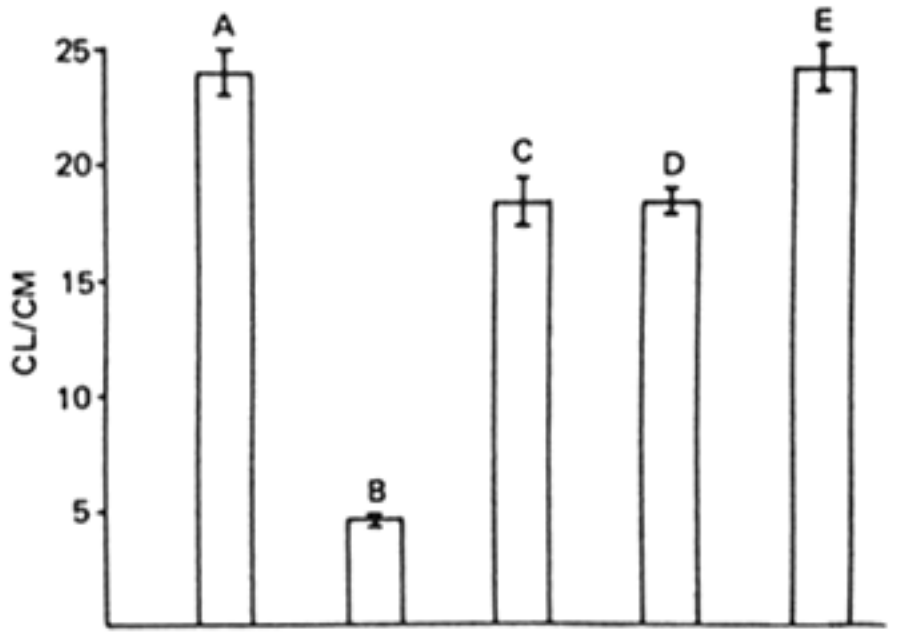

FIGURE 6. Uptake of rubidium by rat lens in culture. Lenses isolated from rats were incubated in TC 199 medium containing 2.5 mM Ca^{2+} and 27 mM HCO_3^- and pulsed with Rb-86. After 20 hours, the distribution ratio was determined by measuring the radioactivity in the lens and the medium. *A*, Control with or without riboflavin in the dark and without riboflavin in the light; *B*, with riboflavin in the light; *C*, B + SOD, 15 units; *D*, B + ascorbate (2.5 mM); *E*, B + catalase (25 units).[11]

TABLE 2. Uptake of Rb-86 by the Rat Lens Incubated in Light in Medium Containing Riboflavin: Effect of Various Scavengers

Conditions	*n*	× CL/CM	× E/C × 100
Blank control	20	22.0 ± 3.0	
−SOD (control)	20	5.06 ± 2.27	
+8 Units Mn SOD	9	9.19 ± 2.98	166.9 ± 26.9[a]
+20 Units Mn SOD	11	10.04 ± 2.75	198.2 ± 34.8[a]
−Catalase (control)	20	6.5 ± 2.5	
+4 Units catalase	10	20.0 ± 3.00	307.0 ± 80.0[a]
+16 Units catalase	10	21.0 ± 2.00	323.0 ± 80.0[a]
$-Na_3Fe(CN)_6$ (control)	16	7.44 ± 1.43	
+5 μM $Na_3Fe(CN)_6$	8	20.44 ± 2.35	213.2 ± 39.8[a]
+10 μM $Na_3Fe(CN)_6$	8	20.88 ± 3.97	280.2 ± 18.4[a]
$-Na_4Fe(CN)_6$ (control)	10	7.00 ± 1.47	
+5 μM $Na_4Fe(CN)_6$	5	19.55 ± 2.00	
+10 μM $Na_4Fe(CN)_6$	5	19.97 ± 3.86	223.3 ± 15.1[a]
−Mannitol (control)	7	6.02 ± 3.20	
+100 μM Mannitol	8	11.05 ± 3.00	138.9 ± 34.5[a]
$+FeSO_4$ (5 μM) (control)	8	8.33 ± 2.19	
$+FeSO_4$ (5 μM) + 100 μM Mannitol	10	12.09 ± 3.07	156.6 ± 31.3[a]

The blank control consisted of lenses incubated in the dark in medium containing 50 μM riboflavin. In other cases, the controls and corresponding experimentals were incubated in light for a period of 18-20 hours and CL/CM determined. The values have been expressed as mean ± standard deviation.

[a] Values are significantly different from the contralateral controls, the *p* values being < 0.001.[12]

attained following overnight incubation. The uptake of this ion by the lenses incubated exposed to light was substantially lower; no effect on rubidium accumulation was observed in the lenses incubated in the dark. The lower accumulation of the ion represents physiological damage. The damage is clearly photochemical in nature. The addition of catalase to the medium protects the lens against this damage completely, the uptake of rubidium in the presence of this enzyme being identical to that in the dark. Thus, hydrogen peroxide generated by the dismutation of the photochemically produced O_2^- is an important agent of lens injury. The addition of superoxide dismutase and ascorbate also protected the lens significantly. These experiments, therefore, demonstrate very simply the effectiveness of ascorbate against the induction of oxygen-dependent photoinjury to the lens. That the damage to the lens membranes may involve $OH^\cdot$, in addition to O_2^- and H_2O_2, is indicated by more recent experiments wherein the photodamage to the lens cation pump could also be prevented by ferricyanide, ferrocyanide, and mannitol, in addition to superoxide dismutase and catalase (TABLE 2). The protective effect of ferrocyanide has been explained to be due to its interaction with O_2^-, thus removing the latter from the medium of incubation. The interaction follows the following sequence:

	Δ E°′ volts
$O_2^- \rightarrow O_2 + e$	+0.33
$Fe(CN)_6^{3-} + e \rightarrow Fe(CN)_6^{4}$	+0.46
$O_2^- + Fe(CN)_6^{3-} \rightarrow O_2 + Fe(CN)_6^{4-}$	+0.79

The protective effect of the ferrocyanide appears to be due to its reaction with H_2O_2, as well as with $OH^\cdot$. This was indicated from the DMPO-dependent inhibition of the oxidation of ferrocyanide to ferricyanide by H_2O_2, as well as by the protection of the pump damage by mannitol, a hexitol known to scavenge $OH^\cdot$ more widely.[12]

The observed protective effect of ascorbate against the photosensitized damage to the lens cation pump led us to investigate the significance of ascorbate in relation to oxygen damage to the lens in greater detail. In experiments described in FIGURE 7, lens incubations were conducted in the medium TC 199 without any additional amount of riboflavin and the photodamage was assessed in terms of lipid peroxidation[13] as apparent by the level of malonaldehyde (MDA) formed by the series of reactions shown in FIGURE 7. As summarized in FIGURE 8, the physiological level of this aldehyde in the lens is substantially low. Incubation in the dark did not affect this level. However, MDA content of the lenses incubated in the presence of light was observed to be substantially greater than that of the lenses incubated in the dark. Since the elevation in the level of MDA took place in the medium without any additional amount of photosensitizers over and above that normally present in the culture medium, peroxidative degradation of the lens membrane lipids as a contributing factor in the genesis of cataracts appears quite feasible. The composition of TC 199 is close to that of the aqueous humor and the light intensity used in these experiments is within physiological limits.

Again, addition of superoxide dismutase, catalase, and physiological amounts of ascorbate to the culture system prevented the light-catalyzed formation of MDA. Since superoxide dismutase and catalase would not enter the cell, the damage to the lens lipids appears to be localized at the membrane level. The protective effect of ascorbic acid is apparently due to its interaction with O_2^-, H_2O_2, as well as $OH^\cdot$, all of which are supposed to react with ascorbate with different rate constants. (TABLE 3).

A tissue organ cultured in the presence of light can, however, be structurally altered irrespective of the generation of the active species of oxygen because of the

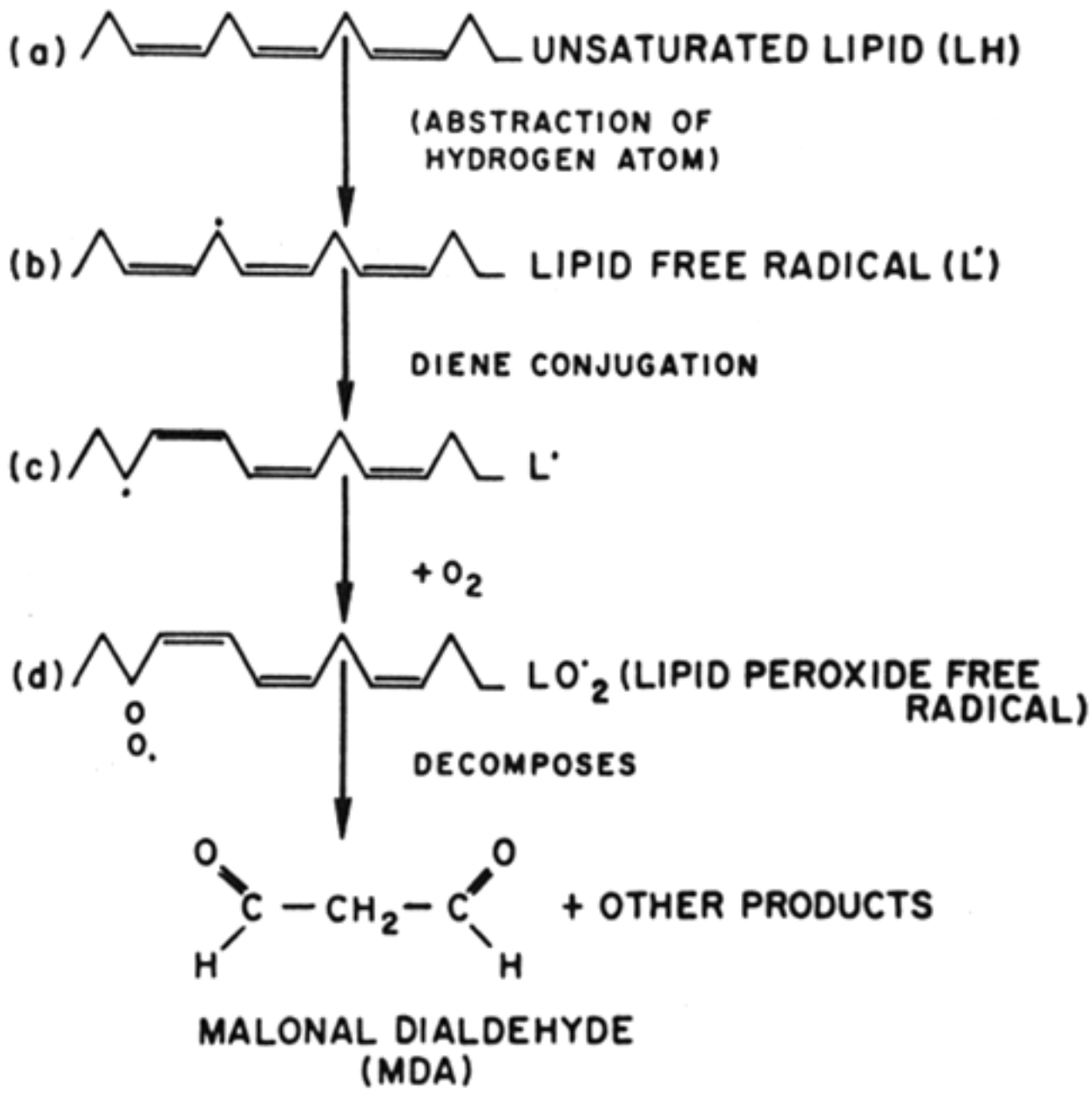

FIGURE 7. Peroxidative degradation of unsaturated lipids. Malonaldehyde is one of the final products.

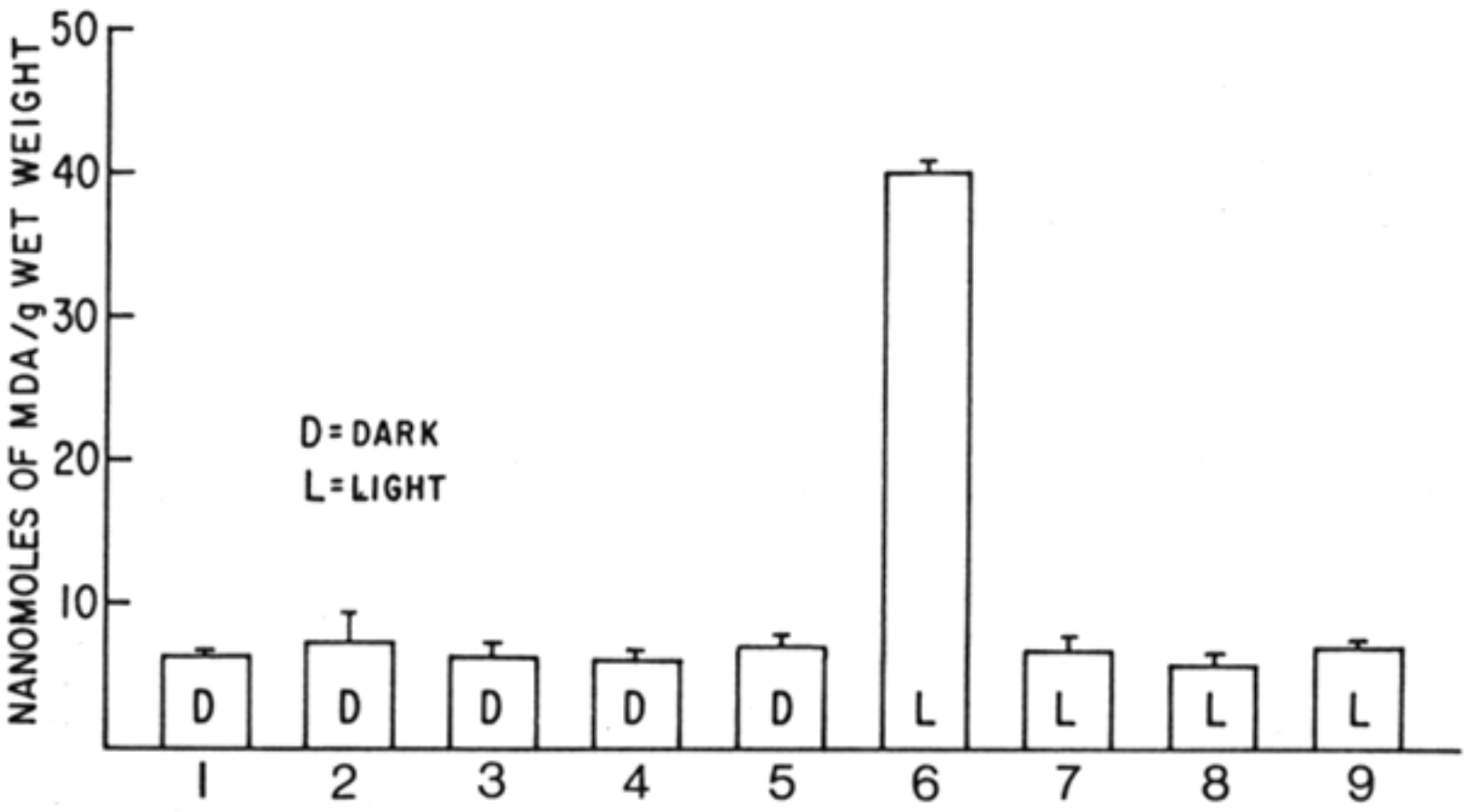

FIGURE 8. Malonaldehyde content of rat lenses freshly dissected and following incubation. The incubation conditions were similar to those described in FIGURE 6, except that the medium lacked riboflavin. MDA was determined in the trichloroacetic acid lens extract by treating the latter with 2-thiobarbituric acid.[13] 1, Freshly dissected unincubated lens; 2, basal medium (BM) in dark (D); 3, BM + SOD (D); 4, BM + catalase (D); 5, BM + ascorbate (D); 6, BM + light (L); 7, BM + SOD (L); 8, BM + catalase (L); 9, BM + ascorbate (L).

endogenous photosensitizers. This oxygen-independent structural alteration may render the tissue more susceptible to damage by the various active species of oxygen. It was, thus, considered appropriate to culture the tissue also in a system producing active species of oxygen by nonphotochemical means and then examine the moderating effect of ascorbate, if any. A xanthine/xanthine oxidase system was used for this purpose. The action of xanthine oxidase on xanthine in the presence of oxygen leads to the formation of all the important active species of oxygen, *i.e.*, O_2^-, H_2O_2, and $OH^\cdot$.[14] The xanthine/xanthine oxidase system thus offers the opportunity of testing the efficacy of an antioxidant in a more challenging situation. Lenses were thus incubated in the presence of xanthine and xanthine oxidase generating H_2O_2 at the rate shown in FIGURE 9, and rubidium uptake determined in the absence and presence of ascorbate.[15]

FIGURE 10 describes the accumulation of the rubidium by the lens as a function of time. In the absence of xanthine oxidase, the uptake follows an approximately linear course until 2 hours. Subsequently, the process becomes curvilinear, tending towards

TABLE 3. The Energetics of Ascorbate Reaction with Xanthine Oxygen Interaction Products Generated by Xanthine Oxidase

	Δn E° (Volts)	$\Delta G°'$ (kcal/mol)	Rate Constant
$HA^- \longrightarrow A + H^+ + 2e$	−0.108		
$H_2O_2 + 2H^+ + 2e \longrightarrow 2H_2O$	+2.640		
HA^- [a] $+ H_2O_2 + H^+ \rightarrow A + 2H_2O$	2.532	−58.4	8 $M^{-1}s^{-1}$
$HA^- \longrightarrow A^{\cdot -} + H^+ + e$	−0.282		
$OH^\cdot + H^+ + e \longrightarrow H_2O$	+2.180		
HA^- [b] $+ OH^\cdot \longrightarrow H_2O + A^{\cdot -}$	1.898	−43.8	1.3×10^{10} $M^{-1}s^{-1}$
HA^- [c] $+ O_2^- \longrightarrow$ products		−15.2	5×10^4 $M^{-1}s^{-1}$

[a] Determined in the author's laboratory.[12]
[b] Farhataziz and Ross.[22]
[c] Cabelli and Bielski.[23]

an asymptote approaching the steady state. The time course and the magnitude of accumulation remained virtually unaffected if xanthine was eliminated from the medium. Thus, xanthine, over the time period, did not affect the physiology of the lens as reflected by its cation transport activity. The addition of xanthine oxidase to the xanthine-containing medium, however, exerted a toxic effect on the lens. This was evidenced by the decrease in the uptake of rubidium, observed at all time periods. The decrease was more substantial with 0.1 unit than with 0.01 unit of the enzyme. Subsequent experiments were conducted using 0.1 units of xanthine oxidase.

The incorporation of 2 mM sodium ascorbate in the xanthine/xanthine oxidase containing medium led to a greater accumulation of rubidium as compared to that in the contralateral control lenses incubated in medium without any ascorbate (FIG. 11). At the end of a 3-hour incubation experiment, the distribution ratio attained in the presence of ascorbate was approximately twice that in its absence. The overall rate of accumulation was also greater in the presence of ascorbate. The effect of varying

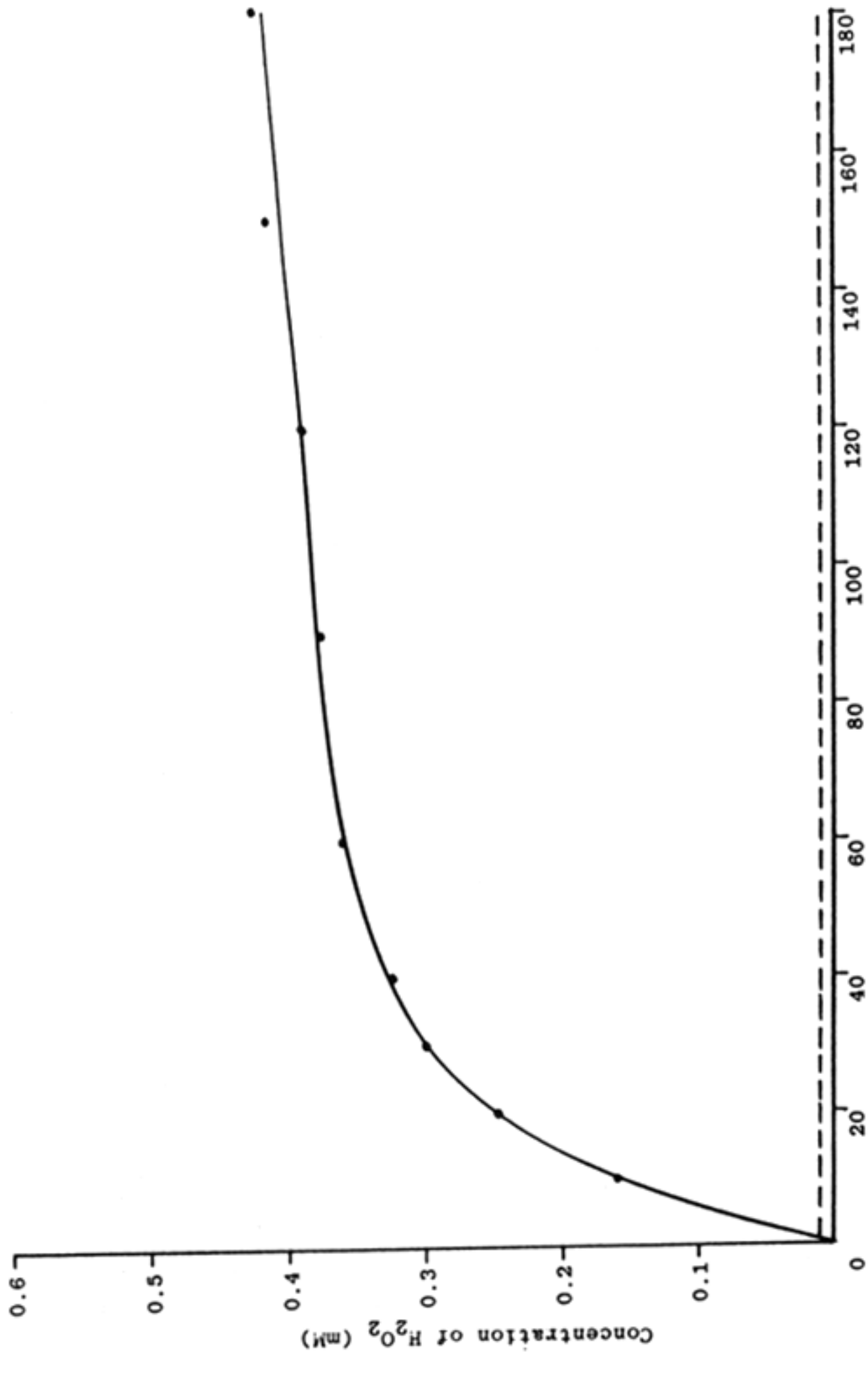

FIGURE 9. Hydrogen perioxide generation in the incubation medium: xanthine (1 mM), xanthine oxidase (0.1 unit/4 ml). The incubation medium was tyrode with Mg^{2+} and contained 5.5 mM glucose. The dotted line close to the abscissa indicates traces of H_2O_2 in the medium incubated with ascorbate (2 mM). H_2O_2 was determined iodometrically; 0.5 ml of the medium was mixed with 0.1 M acetate buffer pH 3.5, 1 ml of 0.6 M KI, 0.5 ml of 1.7×10^{-3} M, ammonium molybdate, and 0.5 ml of 1% starch. The liberated iodine was titrated with 0.5 mM $Na_2S_2O_3$. In the medium incubated with ascorbate, the peroxide determination was done after reacting 0.5 ml of the medium with 20 units of ascorbate oxidase for 15 minutes.

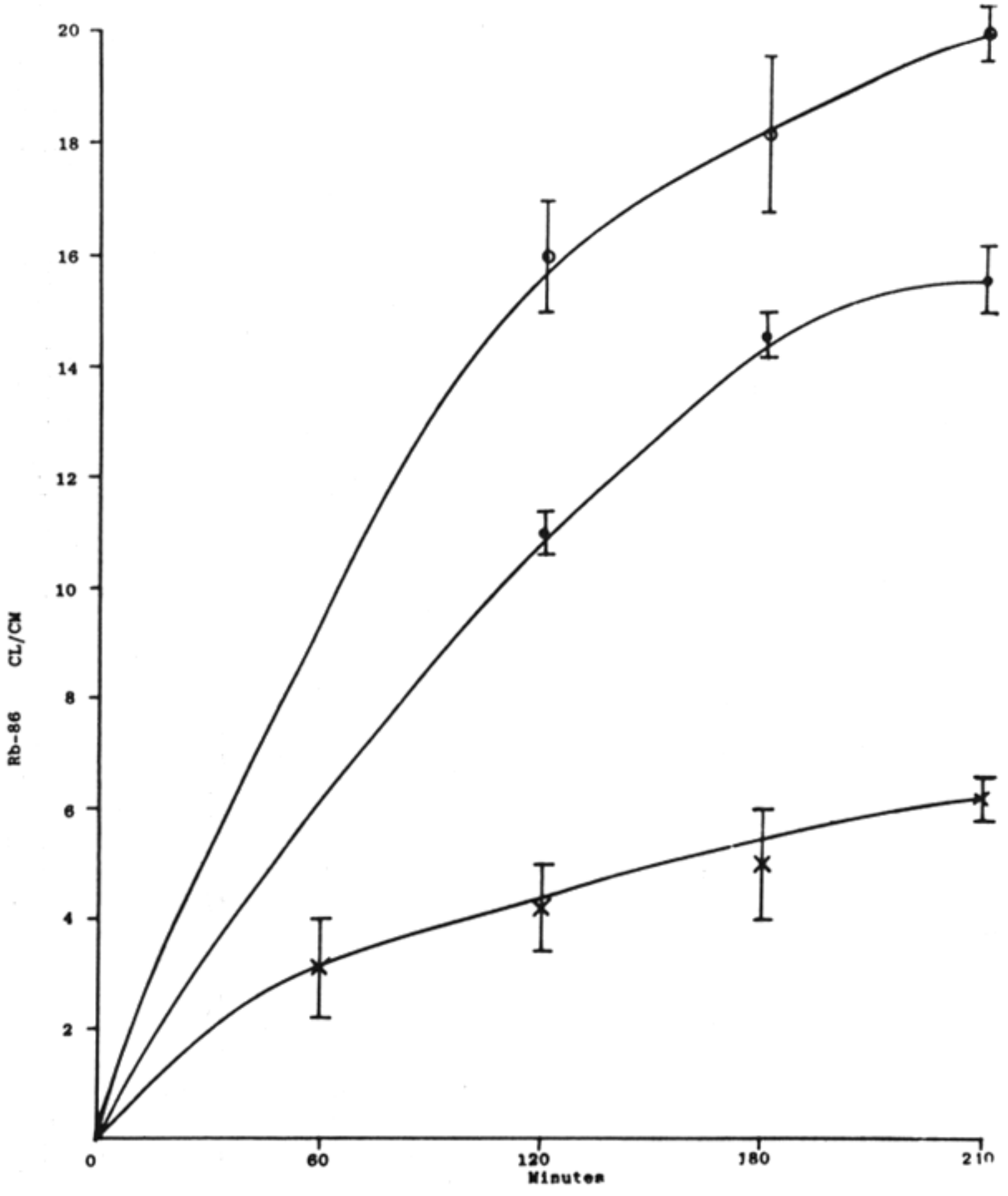

FIGURE 10. Effect of xanthine/xanthine oxidase system on Rb-86 accumulation by rat lens. Lenses were incubated at 36°C in tyrode medium without Mg^{2+} containing 5.5 mM glucose and 1 mM sodium xanthine. Xanthine oxidase/4 ml of incubation medium; ○ = 0 unit; ● = 0.01 unit; × = 0.1 unit. Incubator was gassed with oxygen/air mixture (95/5). CL/CM = concentration of rubidium in the lens/concentration of rubidium in the medium of incubation.

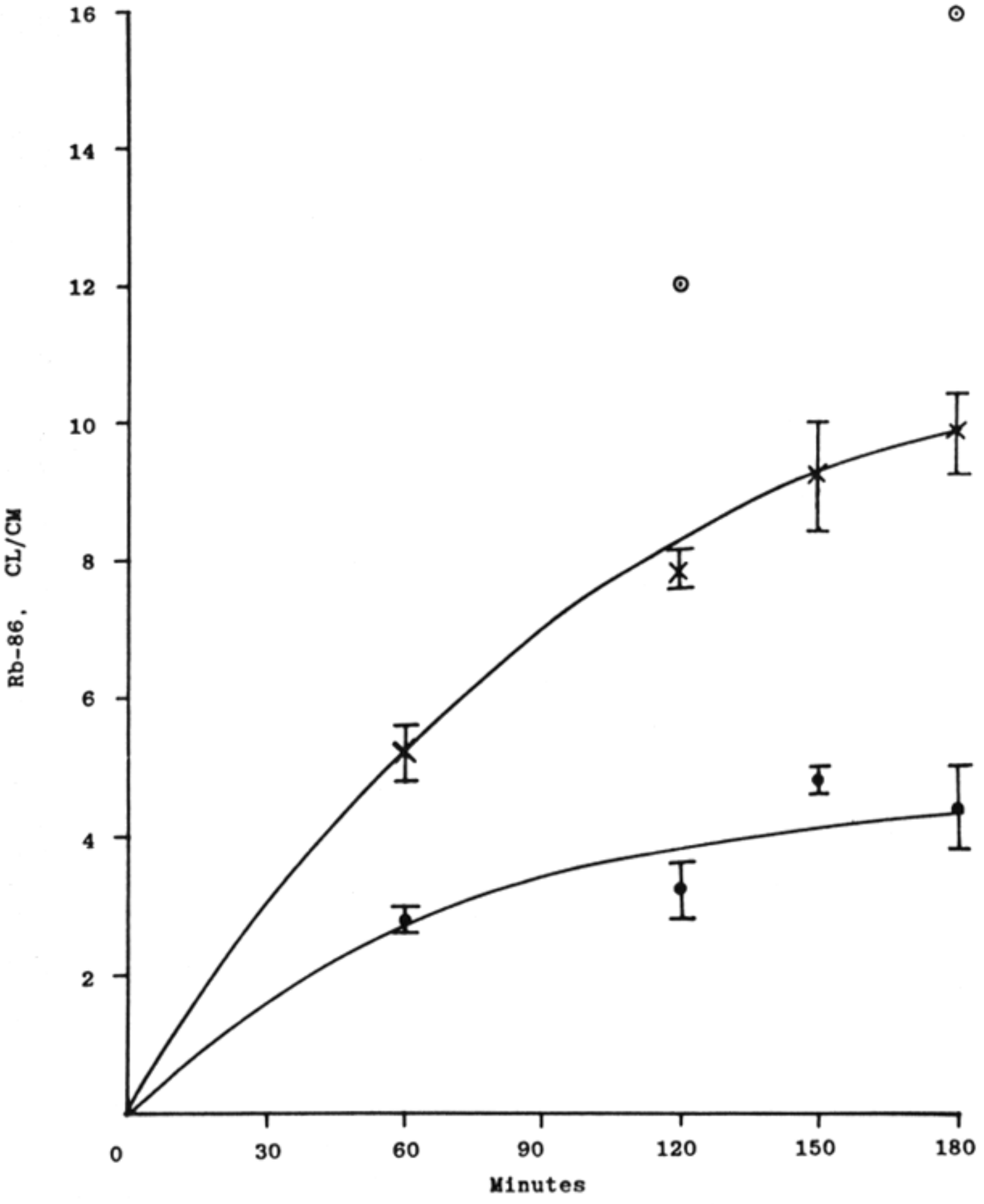

FIGURE 11. Uptake of rubidium in rat lenses incubated in medium containing xanthine (1 mM) and xanthine oxidase (0.1 unit/4 ml of medium): effect of ascorbate. Ascorbate concentration: ● = 0, × = 2 mM. Blank controls (⊙) consisted of lenses incubated without xanthine oxidase and ascorbate. The values corresponding to 60, 120, and 150 minutes represent mean ± standard deviation of three experiments. The values at 3 hours represent mean ± standard deviation of six experiments. Contralaterally paired lenses were used.

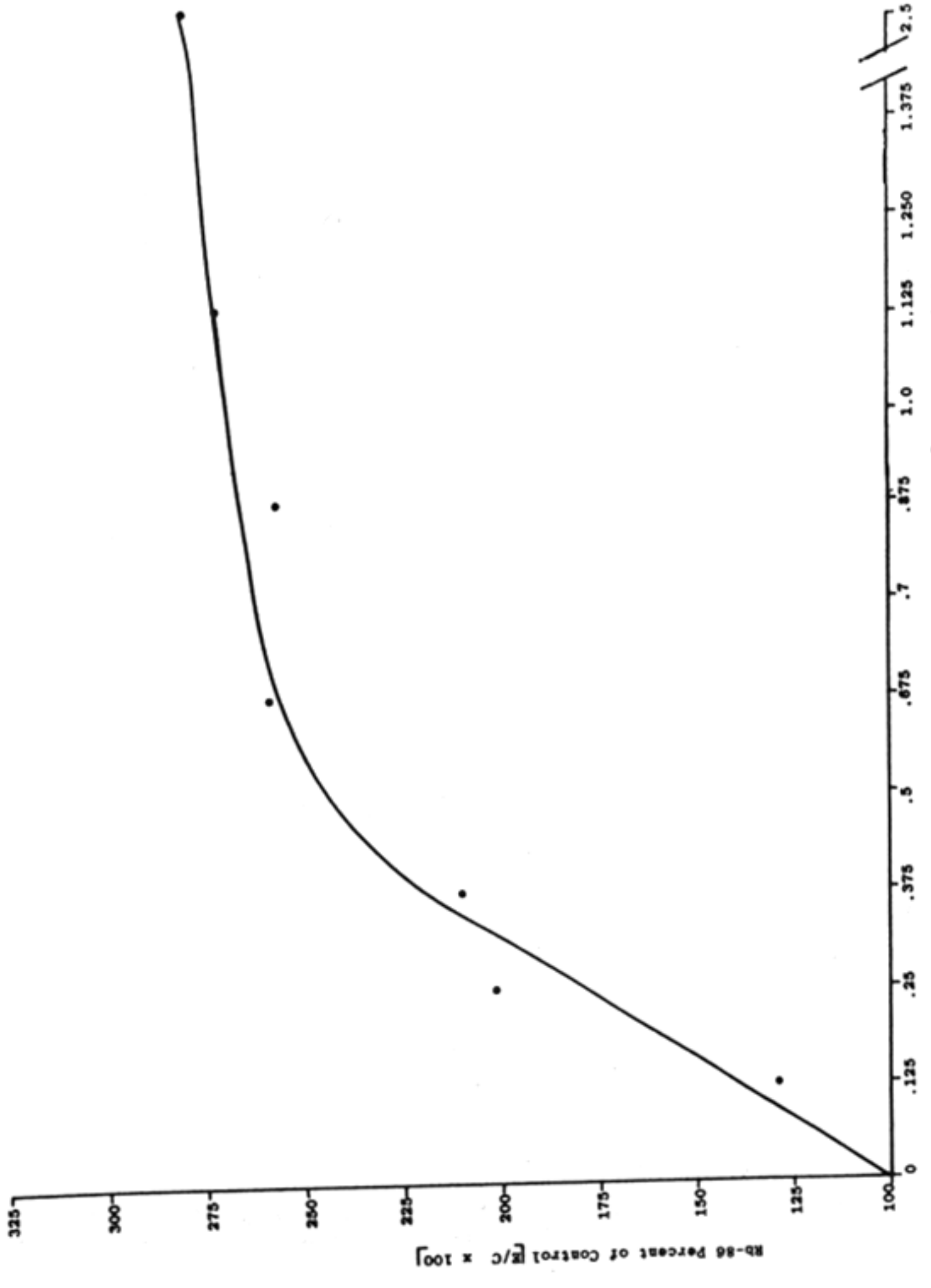

FIGURE 12. Effect of varying concentrations of ascorbate on Rb-86 uptake. Rat lenses were incubated in the presence of medium containing xanthine (1 mM) and xanthine oxidase (0.1 unit/4 ml) as described in FIGURE 10. The results are expressed as E/C × 100, E being the uptake by the lens in the presence and C being the uptake in the absence of ascorbate.

concentrations of ascorbate on the uptake of rubidium by the lens has been described in FIG. 12. Here the results are expressed as the ratio between the rubidium contents of the lenses incubated in the presence and absence of ascorbate multiplied by 100 (E/C × 100). A value above 100 indicated the protective effect. The protective effect of ascorbate was approximately linear up to 0.5 mM. Subsequent increases in ascorbate did not cause a commensurate increase in the protective effect.

The time courses of α-aminoisobutyric acid transport from the medium containing xanthine and xanthine oxidase in the absence (control) and presence (experimental)

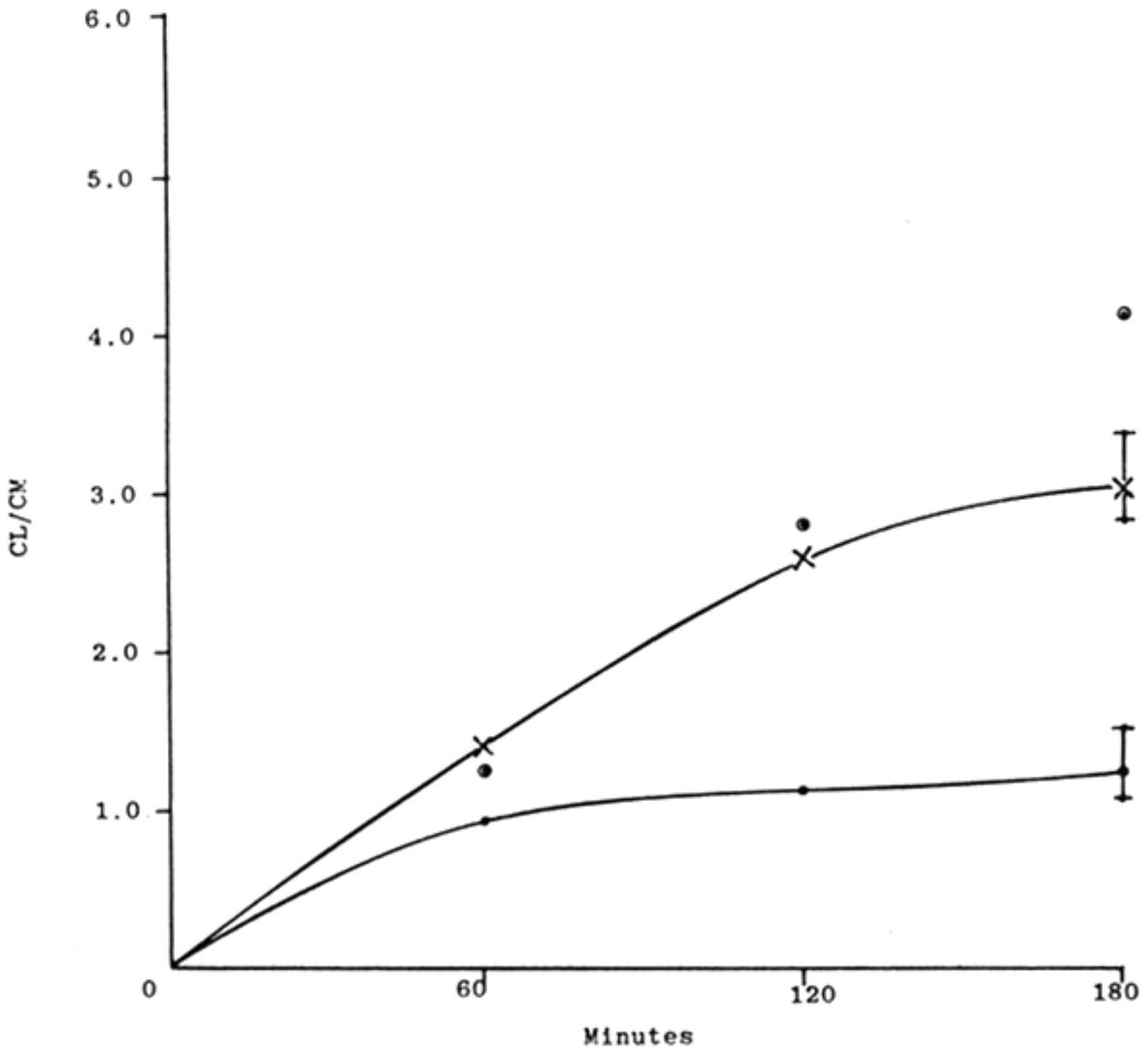

FIGURE 13. Uptake of [^{14}C]AIB in rat lenses. Medium of incubation was tyrode with Mg^{2+}: glucose (5.5 mM), sodium xanthine (1 mM), xanthine oxidase (0.1 unit/4 ml). Gas was O_2:CO_2, (95:5). Points at 60 and 120 minutes are derived from single contralateral pairs. Points at 180 minutes represent three contralateral pairs. ● = No ascorbic acid; × = ascorbate (2 mM); ⊙ = blank controls, representing duplicate incubation experiments without xanthine oxidase and without ascorbate.

of sodium ascorbate along with the blank controls have been described in FIGURE 13. Akin to the case of rubidium, the magnitude as well as the rate of AIB uptake was greater in the presence of ascorbate and compared favorably with that observed in the blank control experiments conducted in the medium without xanthine oxidase.

Data for comparison between various groups were obtained by 3-hour incubation experiments. As described in TABLE 4, ascorbate by itself has no effect on Rb^+ uptake

by the lenses incubated in the basal medium without xanthine/xanthine oxidase. Also, dehydroascorbate had no protective effect, either in terms of rubidium or AIB uptake. Ascorbate is indeed able to protect the lens even in the oxidizing medium generated by xanthine/xanthine oxidase, and it is quite possible that it may be able to perform a similar antioxidant function *in vivo* also.

Ascorbate, however, should not be an exception in protecting the lens against oxidative damage. It is most effective probably because of the existence of the ascorbate pump and consequent maintenance of its high concentration in the aqueous and the lens. Vitamin E, another nutritional antioxidant, though not known to be actively transported across the blood aqueous barrier, should be equally effective. As sum-

TABLE 4. Effect of Ascorbic Acid on Rubidium and α-Aminoisobutyric Acid (AIB) Uptake[a, d]

Additions to the Medium	CL/CM	E/C × 100	n[c]
	RUBIDIUM UPTAKE		
C[e] none	21 ± 1.5		6
E[f] Na ascorbate (1.0 mM)	21 ± 2.0	100	2
E Na ascorbate (2.0 mM)	21.5 ± 0.5	102	3
C xanthine (1 mM)	19 ± 1.5	24	6
E xanthine + XO[b] (0.1 unit)	4.5 ± 1.0		
C XO (0.1 unit)	5.2 ± 0.5	200	10
E XO + Na ascorbate (2mM)	10.4 ± 0.4		
C XO (0.1 unit)	4.54 ± 0.4	105	5
E XO + DHA (2.5 mM)	4.78 ± 0.28		
	AIB UPTAKE		
xanthine (1 mM)	5.5 ± 0.5		4
C xanthine + XO (0.1 unit)	1.48 ± 0.38	203	7
E xanthine + Na ascorbate (2 mM)	3.0 ± 0.47		
C XO (0.1 unit)	0.92 ± 0.31	108	5
E XO + DHA (2.5 mM)	1.0 ± 0.5		

[a] Paired lenses were incubated as C and E in the medium with additions indicated.
[b] XO = xanthine oxidase.
[c] *n* = Number of contralateral pairs used in each group of experiments.
[d] Rubidium and AIB were used as ^{86}RbCl and C-14 AIB respectively.
[e] C = Control.
[f] E = Contralateral lenses incubated as experimental.[12]

marized in FIGURE 14, low concentrations of this nutrient also inhibit the photoperoxidative degradation of lens lipids quite effectively.[16] Higher concentrations were not prooxidative.

The final test for involvement of oxidation of membrane lipids, membrane proteins, or membrane-bound enzymes in the formation of cataracts must come from a study of the animal model system. Unfortunately, there is a general lack of models of senile cataracts. One that has been recently developed is known as the Emory mouse. These animals are known to develop cataracts by the ninth to eleventh month. More recently,

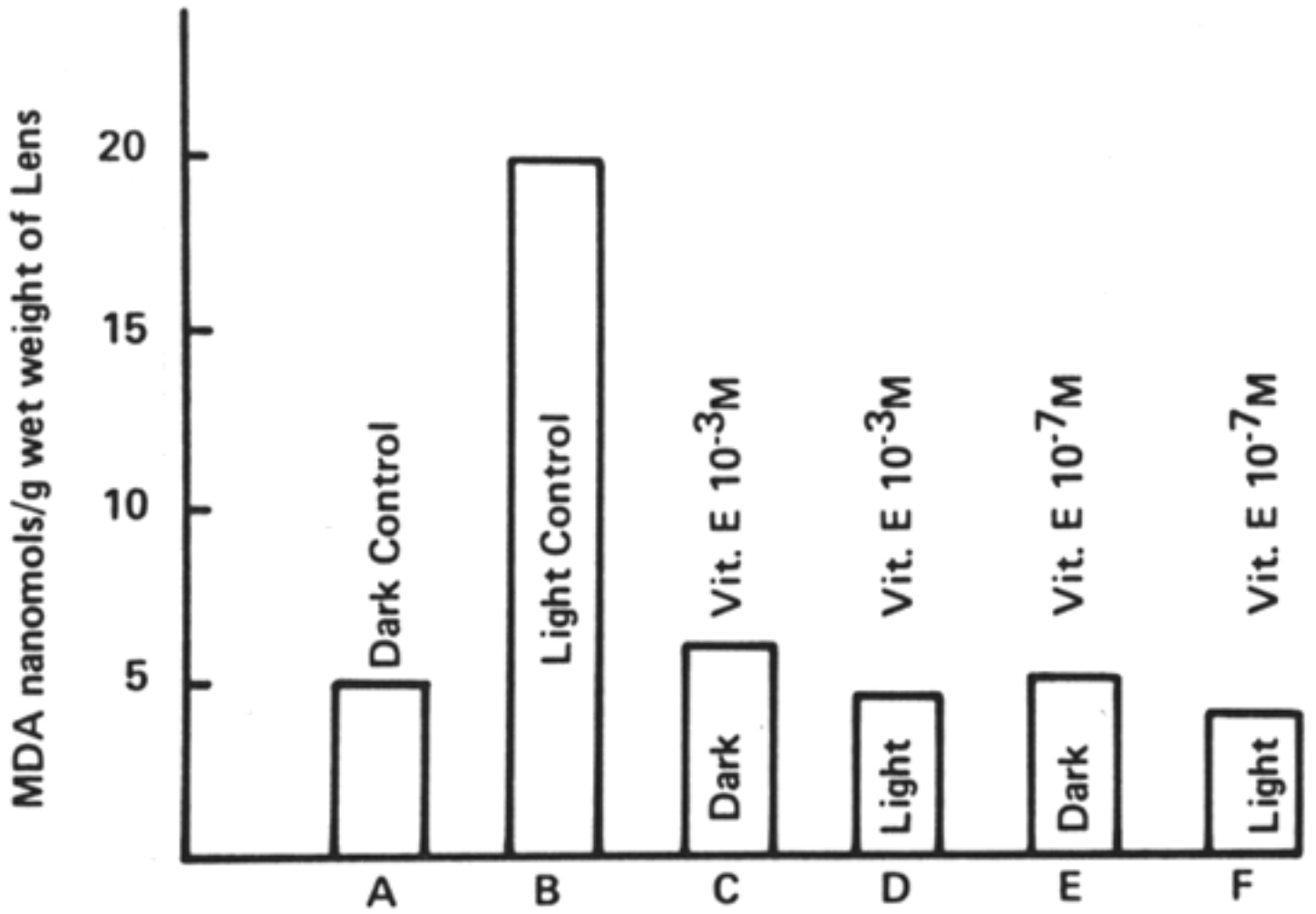

FIGURE 14. Photoperoxidation of rat lens lipids under organ culture conditions: prevention by vitamin E.[16] Period of incubation was 20 hours.

studies from this laboratory demonstrate that an oxidative stress may indeed be one of the contributing factors involved in the development of cataracts in these animals. Six-week-old mice were housed in individual cages and given Purina lab chow ad lib. Animals in one group[17] were given weekly 0.1 ml of coconut oil intraperitoneally as a placebo and designated as the control group. The members of the other group, designated as the experimental group, received intraperitoneally 0.1 ml of the coconut oil containing 10 mg of DL-α-tocopherol acetate. The progress of cataracts, as monitored by an ophthalmoscope and Scheimpflug photography, using 1% homatropine as a pupillary dilator, is depicted in FIGURE 15. The animals were also weighed at

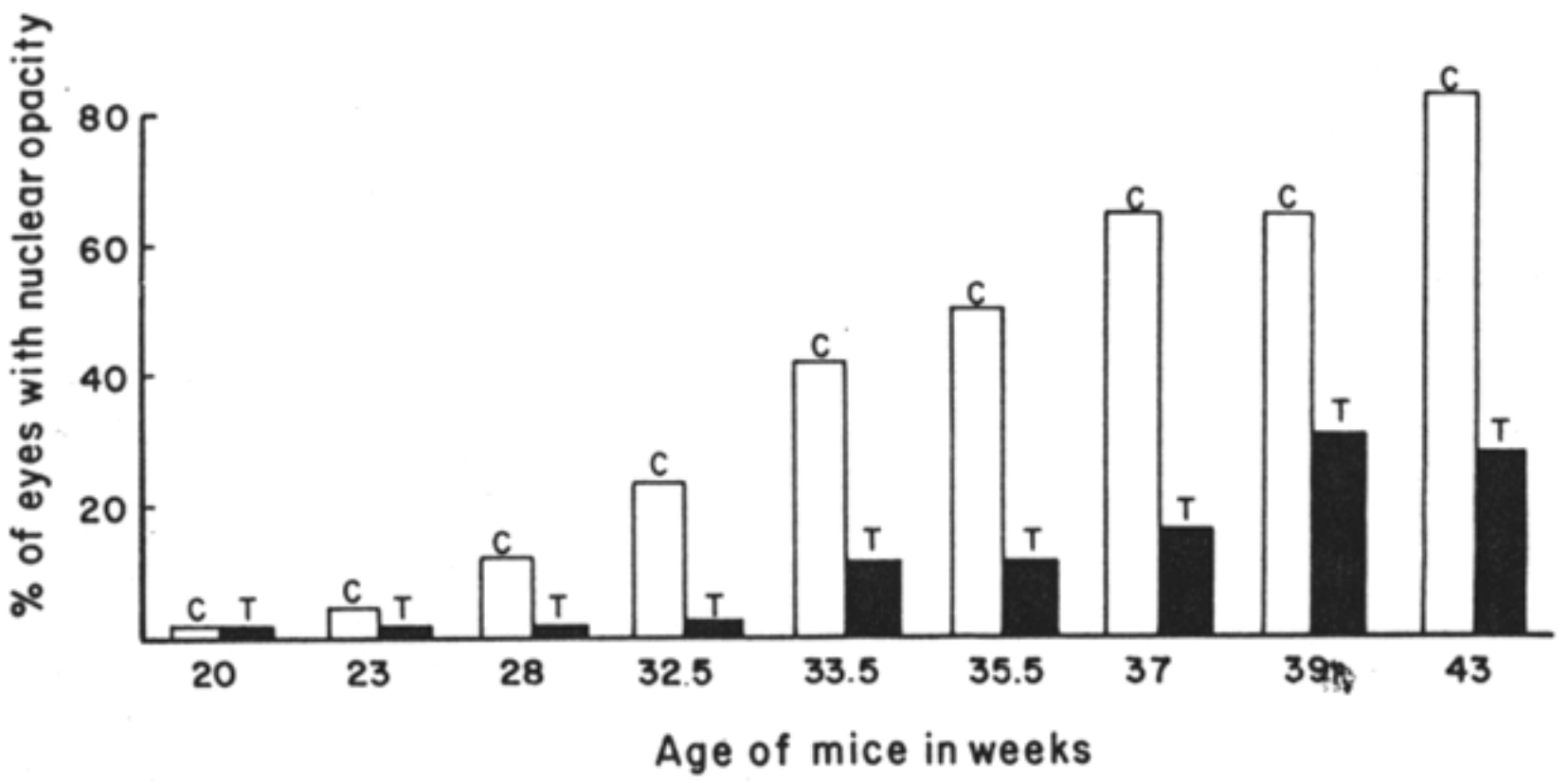

FIGURE 15. Effect of vitamin E on the progress of cataracts in emory mice[17]; *C,* treated with placebo, *T,* treated with vitamin E.

frequent intervals. If the animals were kept on a synthetic diet consisting of casein, starch, coconut oil, vitamins, and salts, the mortality was high. It was, therefore, necessary to maintain them on the usual laboratory diet which is said to contain 30 mg of vitamin E (*dl*-α-tocopherol acetate) per kg. The majority of the animals in the control group, as reported, did develop advanced opacity (nuclear) by about the tenth month. In the experimental group, the percentage of animals having a comparable degree of lens opacity was substantially lower at all periods of examination, indicating that vitamin E had a definite protective effect.[17] Obviously, the amount of vitamin E present in the diet is somehow not available to the animal, or the animal has, for some reason unknown at the present time, an increased vitamin E requirement. Bhuyan *et al.*[7] have reported that the liver of these animals has a higher malonaldehyde level as compared to other laboratory mice. This indicates the mice are suffering from some kind of oxidative stress. The possibility that the release of polyunsaturated fatty acids during retinal degeneration in the early periods of the animal's life might have contributed towards cataract development also remains to be investigated. Nevertheless, the preventive action of vitamin E on cataracts was quite striking, indicating that oxidative stress may indeed lead to cataract formation *in vivo*.

The aforementioned studies on laboratory animals indicate the possibility that the oxidative stress imposed through the generation of oxygen radicals and their subsequent derivatization to other oxidants may initiate or participate in the process of age-dependent cataractogenesis (senile cataracts) in human beings also, light and oxygen, as explained earlier, acting synergistically through some photosensitizers. As mentioned before, these radicals can induce oxidation of proteins, lipids, and other chemical constituents of metabolic and structural importance to the tissue. The oxidation of protein sulfhydryl associated with human cataracts appears now to be well established. Organ culture studies reported above and elsewhere indicate the additional possibility of membrane lipid damage due to the generation of oxidizing radicals and their active derivatives. That such may be the case in human beings is quite possible. Preliminary evidence of lipid damage associated with human cataracts has been obtained by us and others. The content of malonaldehyde was found to be higher in the cataractous lenses as compared to the noncataractous lenses. Furthermore, the level is higher in the brunescent cataracts than in the nonbrunescent cataracts (TABLE 5).[17] Since malonaldehyde is derived by the degradation of the lipid peroxides, the formation of such toxic peroxides with aging and their concomitant role in membrane damage leading towards lens opacification is an interesting hypothesis to consider. These peroxide derivatives can initiate oxidation of protein sulfhydryls while simultaneously altering the membrane structure. Tryptophan residues are also known to be involved.

Malonaldehyde, one of the products of lipid peroxidation, has been shown to act as a cross-linking agent for proteins and phospholipids, yielding complex lipid-soluble fluorescent materials called lipofuscins.[18] The content of such fluorescent material in certain tissues increases with age. Spectrofluorometric studies indicate the presence of lipofuscin-like material in the human lens also. In addition, the human lens has been reported to contain lipid material containing conjugated dienes.[17] These dienes are formed subsequent to the peroxidative degradation of polyunsaturated fatty acid moieties of the tissue lipids. However, data to correlate the contents of lipofuscins, as well as of the conjugated dienes, with aging or cataract formation has not been possible to obtain thus far. Further studies in this direction are in progress.

Whereas the aqueous ascorbate may function to protect the tissue against an oxidative insult to the lens membranes, the significance of a high intracellular level of this nutrient in the lens and other tissues of the eye remains as yet unknown. Mapson and Moustafa[19] observed that in germinating pea seedlings, the dehydroascorbate-ascorbate redox couple is able to facilitate electron transfer from NADPH to

oxygen enzymatically, the GSSG/GSH couple acting as an intermediate electron carrier operating between NADPH and dehydroascorbate (DHA); see FIGURE 16. The possibility of a similar role of the DHA-AA couple also exists in the lens and other tissues of the eye where there is excessive GSH.[20] Therefore, one of the ultimate effects of such an electron transfer may be a stimulation of the hexose monophosphate shunt (HMP shunt). We have recently found this to be true. The activity of the HMP shunt was measured by incubating lenses in the balanced salt medium containing [I-^{14}C]glucose and measuring the CO_2 liberated from utilization of C-1 of glucose. The time course of CO_2 production through the shunt in the presence and the absence of ascorbate and the dehydroascorbate has been described in FIGURE 17. The rate of CO_2 production was higher in the case of the lenses incubated with ascorbate, the enhancement in the rate becoming significant after thirty minutes of incubation. The amount of CO_2 produced by incubation of the lenses for 60 minutes in the presence of ascorbate was approximately twice that produced in its absence. This increase in shunt activity brought about by ascorbate was not abolished by the addition of catalase in the medium. This suggests that the shunt stimulation does not involve, to any

TABLE 5. Malonaldehyde Content in Human Cataracts

Cataract Type	Malonaldehyde (nmol)	
	Per Lens	Per g Wet Lens
Yellow cataract	0.566 ± .04	2.181 ± 0.10 (20)
Brown cataract	0.915 ± .05	3.715 ± 0.30 (18)

Intracapsularly removed cataracts were used. Following surgical extraction, they were quickly weighed and transferred to homogenizing tubes containing 1 ml of 10% trichloroacetic acid solution; 0.5 ml of the protein free supernatant obtained after centrifugation was mixed with 0.5 ml of 0.71% L-thiobarbituric acid solution. The mixture was heated in boiling water bath for 15 minutes and MDA values calculated from the optical density of the resulting solution at 532 nm. Tetramethoxy propane was used as a standard. The number of samples is given in parentheses. The values are expressed as mean ± standard error; p value between brown and yellow cataracts is < 0.01.[17]

significant extent, hydrogen peroxide that might be generated in the incubation medium during the oxidation of ascorbate. The concentration of the ascorbate in the medium following 1 hour of incubation remained approximately 95% of the starting concentration. No peroxide could be detected. The possibility of its decomposition to water because of the excess of ascorbate cannot be ruled out. An alternate possibility, that the stimulation of the shunt may be due to the dehydroascorbate derived by ascorbate oxidation, was examined next. In accordance with this prediction, addition of DHA to the medium stimulated the shunt very substantially. The amount of CO_2 liberated by the lens when incubated in the presence of DHA was approximately 4 to 10 times that in its absence (FIG. 17). It was also about 4 to 5 times over that produced by the lenses incubated in the presence of similar levels of ascorbate.

Subsequent experiments were designed to investigate whether the stimulation of the shunt by the oxidized and the reduced forms of the vitamin C is related to the coupling of the DHA-AA pair with GSSG-GSH pair on the one hand and the oxygen-H_2O pair on the other hand, oxygen serving as the final acceptor of the reducing equivalents. The concentration of glutathione in the tissue is maintained constant due

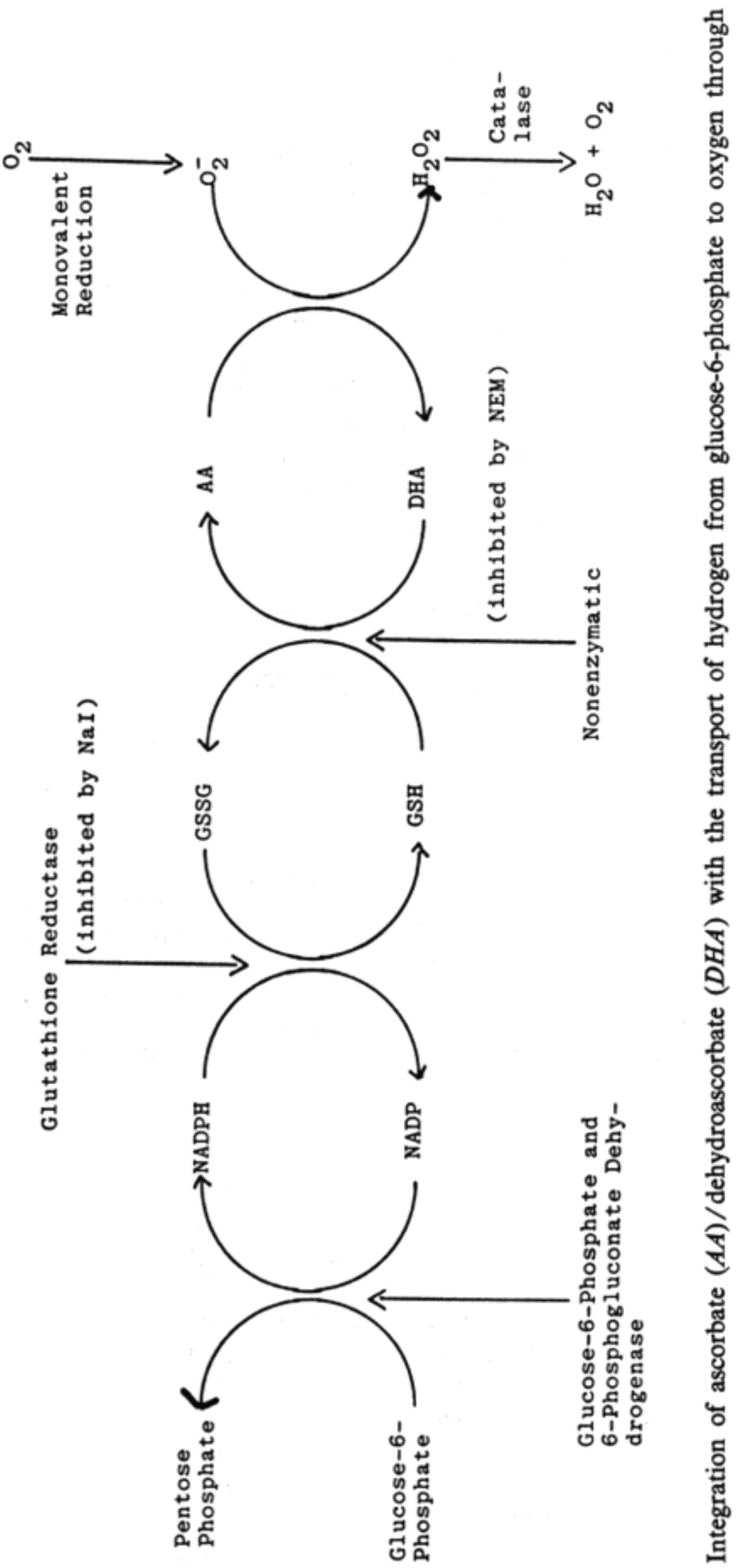

FIGURE 16. Integration of ascorbate (*AA*)/dehydroascorbate (*DHA*) with the transport of hydrogen from glucose-6-phosphate to oxygen through NADPH.

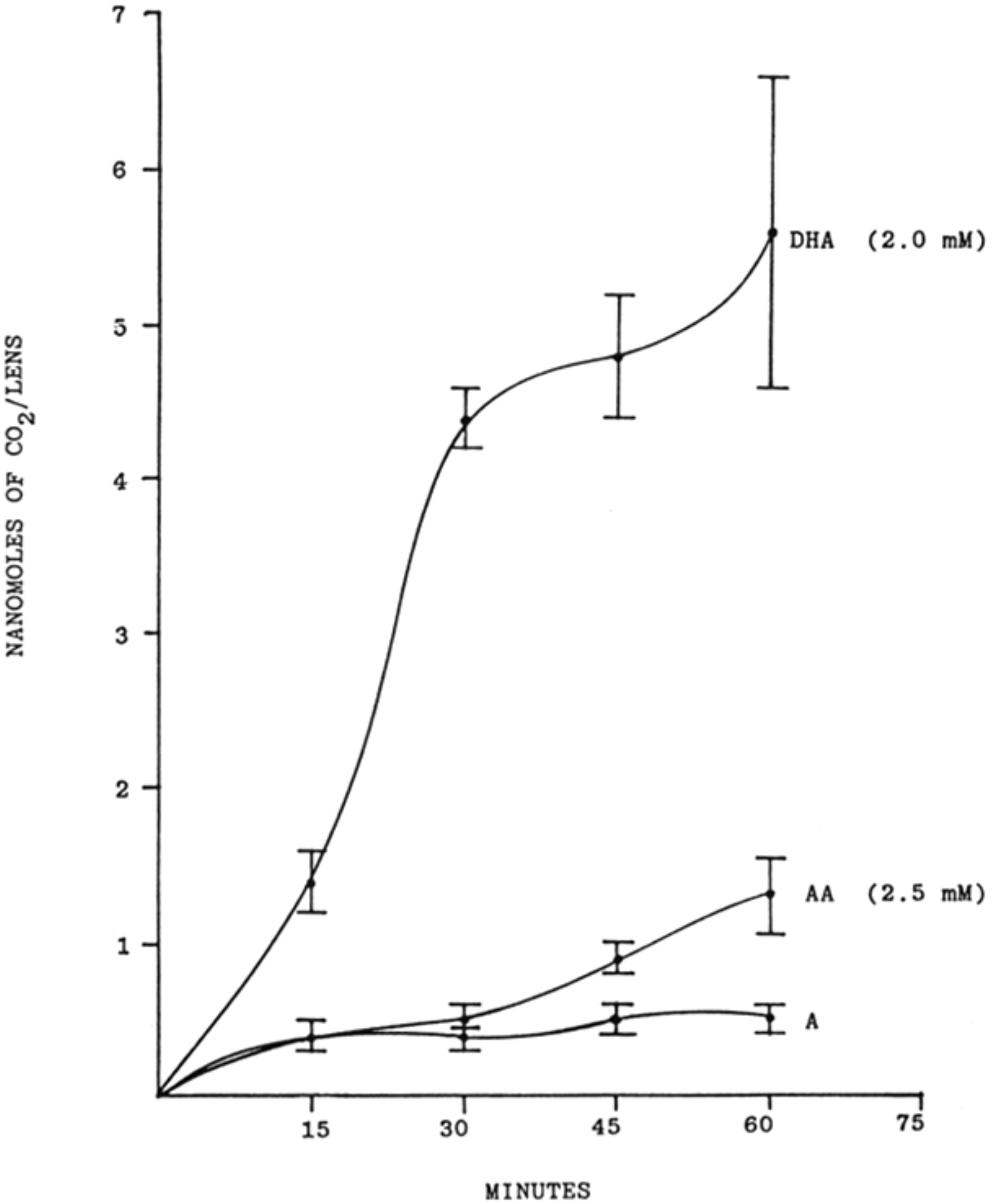

FIGURE 17. Hexose monophosphate shunt in rat lens: effect of ascorbate and dehydroascorbate. The utilization of glucose by the rat lenses through the hexose monophosphate shunt was measured in terms of CO_2 produced by isolated lenses incubated at 37°C in the medium pulsed with [1-^{14}C]glucose. *A*, basal medium; *AA*, medium containing ascorbic acid; *DHA*, medium containing dehydroascorbic acid. Each point represents a mean of four experiments ± standard deviation. The basal medium consisted of tyrode without Mg^{2+}. The glucose concentration was 5.5 mM. The specific activity of ^{14}C was 3.5 μCi/μmol of glucose. Incubations were conducted in test tubes containing a sidearm closed with a rubber stopper. After introducing the lens, the test tube was closed with a stopper through which was hung a plastic cup containing filter paper soaked in hyamine hydroxide (0.1 N). At the end of the incubation, 100 μl of a 10% solution of trichloroacetic acid was injected through the sidearm and CO_2 collected in the hyamine-containing cup by incubating the tubes for another 30 minutes. The plastic cup was then transferred to LSC fluid and radioactivity determined.

to the continued reduction of GSSG by NADPH produced by the HMP shunt. The reduction reaction is catalyzed by glutathione reductase. HMP shunt measurements were thus conducted in the presence of sodium iodide. This compound is a known inhibitor of glutathione reductase.[19] Addition of the iodide to the incubation medium in concentrations ranging from 0 to 4 mM was observed to antagonize the stimulation of the shunt brought about by ascorbate, as well as by dehydroascorbate (FIG. 18). The accelerating effect of ascorbate and dehydroascorbate can thus be attributed to the ability of the cell to continue to maintain glutathione at an appropriate level. The importance of the reduced glutathione was additionally apparent from the effect of NEM on the HMP shunt (FIG. 19). The addition of this compound, in concentrations ranging from 0 to 4 mM, also led to an abolition of the ascorbate and dehydroascorbate stimulating effects. The iodide and NEM effects thus indicate that the DHA- or AA-dependent stimulation of the shunt involves glucose, NADPH, GSSH/GSH, and very likely oxygen. Direct oxygen consumption measurements by the tissue due to the small size of the rat lenses incubated in microliter volumes of medium could not be feasible.

As summarized in the APPENDIX, the potential difference of the reduction of DHA by GSH at pH 7 and 37° C is approximately 0.284 V. The free energy change (ΔG°)

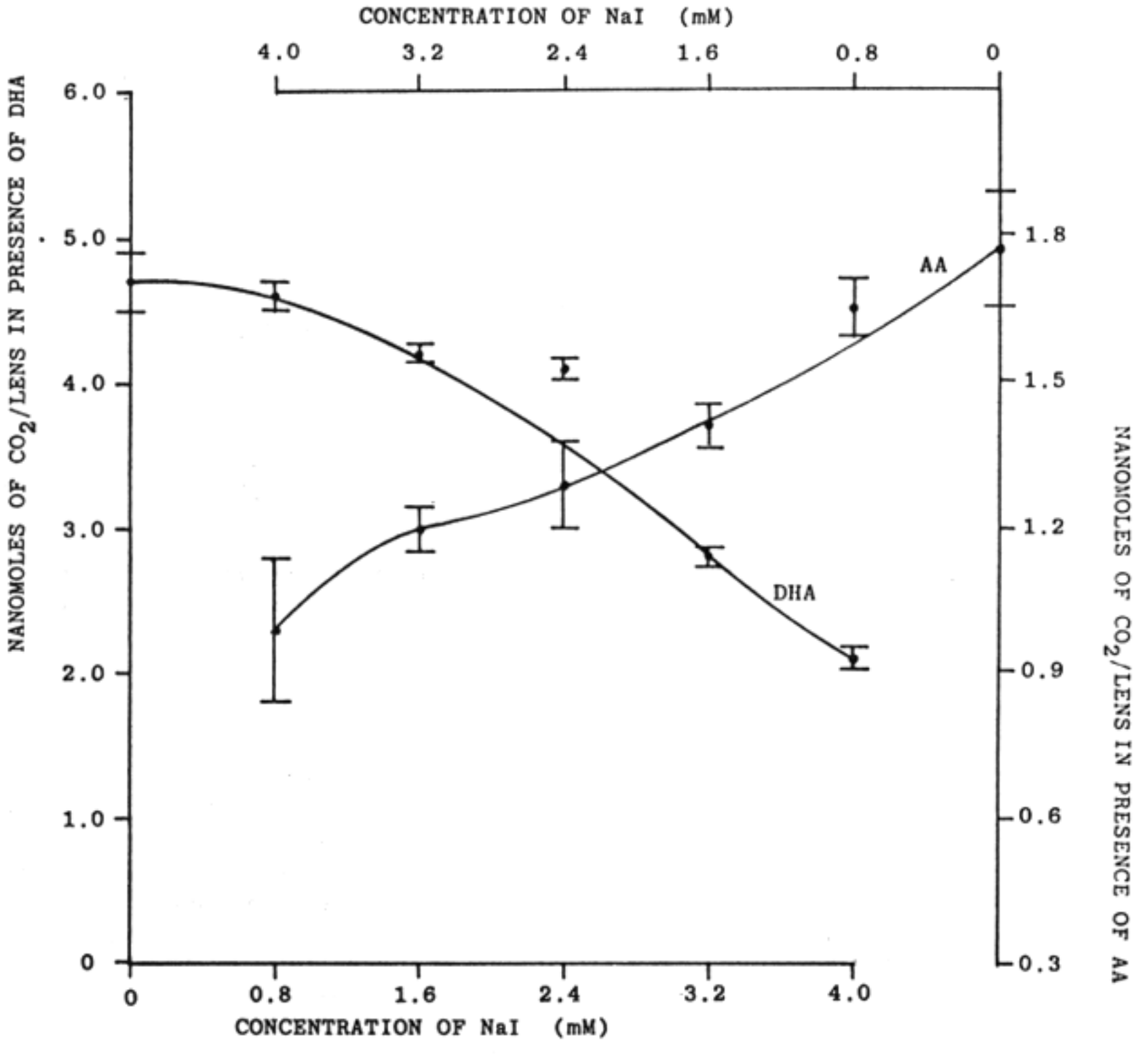

FIGURE 18. Effect of NaI on the production of CO_2 from [1-^{14}C]glucose in the presence of DHA (2 mM) and AA (2.5 mM). Experimental techniques were similar to those described in the legend of FIGURE 17. Each point represents a mean of three experiments ± standard deviation.

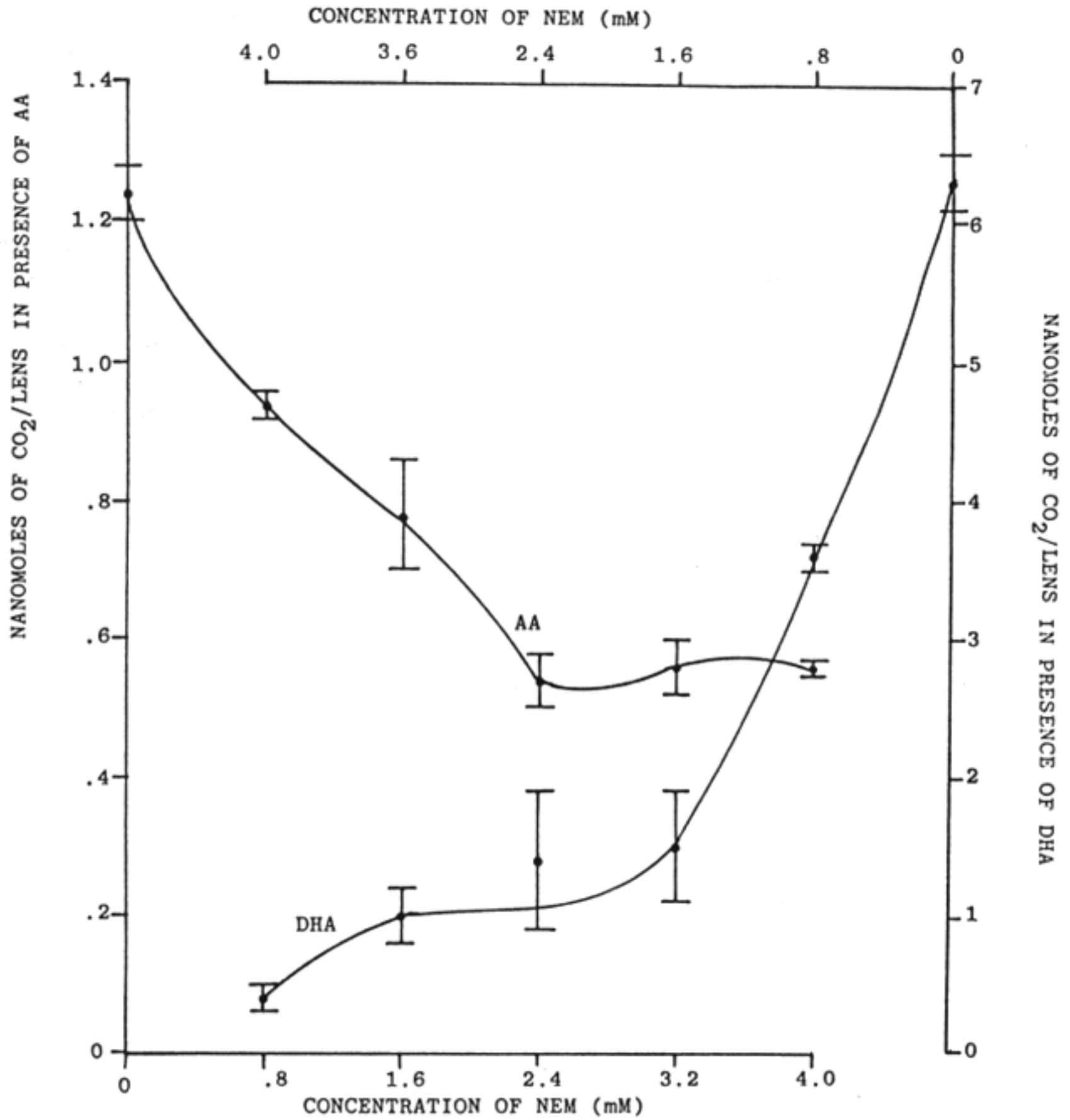

FIGURE 19. Effect of NEM on the production of CO_2 from [1-^{14}C]glucose in the presence of AA (2.5 mM) and DHA (2 mM). The experimental details are similar to those described in FIGURE 17. Each point represents a mean of three experiments ± standard deviation.

associated with the reaction and the theoretical equilibrium constant values are −13 kcal and 1.7×10^9, respectively. The values of the equilibrium constant of the reaction derived from the Gibbs as well as the Nernst equations are identical. These calculated parameters point out that the reduction of DHA by glutathione under standard conditions is thermodynamically quite feasible. The observed acceleration of the shunt by DHA is thus in accordance with the energy considerations of the overall reaction. Based on the equilibrium constant calculations under standard conditions, the ratio of AH_2/DHA is 1.7×10^8. Although the precise relationship between the steady-state concentrations and equilibrium concentrations remains somewhat disputed for most reactions in the biological milieu, the values derived form the equilibrium constant attainable under standard conditions do provide a reasonably good measure of approximation. In the present case, the AH_2/DHA ratio, as calculated under standard

states, agrees with the fact that the DHA level in the lens is below the ordinary limits of detection, whereas AH_2 is about 3 mg/kg wet weight of the tissue. The reactions in the direction indicated in FIGURE 16, therefore, appear kinetically so favorable that the suggested electron flux may proceed in many types of animal cells, despite the absence of dehydroascorbate reductase and ascorbate oxidase. The ascorbate-dehydroascorbate couple may thus be of significant importance in the dynamic regulation of glucose metabolism through the shunt. The physiological significance of the ascorbate-driven electron transport process described above is not quite apparent at the present time, except perhaps in the prevention against oxidative stress to the tissue concomitant to an *in situ* generation of active species of oxygen. The acceleration of the shunt is another way to get rid of the peroxide that is generated following dismutation of O_2^-. It should, however, be mentioned that ascorbate can act in certain circumstances as an oxidant and the possibility of its participation in cataractous process, although unlikely, cannot be entirely ruled out.

TABLE 6. Ascorbic Acid Content of Blood, Aqueous Humor, and Lens

Age in Years	Type of Cataract	Blood (mg/100 ml)	Aqueous Humor (mg/100 ml)	Lens (mg/100 ml)	Group
55.28 (64) ± 10.16	cortical	1.735 (60) ± 1.136	14.0 (52) ± 8.05	31.81 (64) ± 10.8	A
63.75 (20) ± 9.3	nuclear	2.10 (20) ± 1.43	28.3 (12) ± 5.26	30.73 (20) ± 7.55	B
p values between A and B		< 1.22	< 0.001*	< 0.5	

Values have been expressed as mean ± standard deviation; *p* values with *asterisk* are significant. Numbers in parentheses indicate the number of samples in each case.[20]

HUMAN CATARACTS AND VITAMIN C

The implication of ascorbate in human lens metabolism and cataract is very difficult to examine. It was, however, intriguing to find in a limited number of individuals that the ascorbate level in the aqueous humor of patients with cortical cataracts is significantly lower than that in the aqueous humor of patients with nuclear cataracts,[20] the level in the blood and the lens (cataract) not being different (TABLE 6). The blood-to-aqueous ratio of ascorbate in the case of patients with cortical cataracts is approximately 1:7, whereas in patients with nuclear cataracts it was 1:14. This lowering of the ratio in the case of cortical cataracts points out that blood aqueous ascorbate pump may be more sluggish in patients who develop cortical cataracts. It may also be coincidental with the incidence of cortical cataract. The final answer to the question of the role of ascorbate in fighting cataracts must, therefore, await further experimental studies. The importance of ascorbate in another part of the eye, the retina, also must be studied. Recent studies from this laboratory demonstrate that the structures of the neural retina undergo severe degeneration in scurvy.

APPENDIX

$$\begin{array}{rll} & & \Delta E^{\circ\prime}\ (\text{pH } 7.0) \\ 2\ GSH \longrightarrow & GSSG + 2H^{+} + 2e & 0.23\ V^{23} \\ DHA + 2H + 2e \longrightarrow & AH_2 & 0.054\ V^{24} \\ \hline DHA + 2GSH \longrightarrow & GSSG + AH_2 & \Delta E^{\circ\prime} = 0.284 \end{array}$$

$$\begin{aligned} \Delta G^{\circ\prime} &= -(n)\,(F)\,(\Delta E^{\circ\prime}) \\ &= -(2)\ 23.06\ \frac{\text{kcal}}{\text{mol }^{\circ}\text{K V}} \times 0.284\ \text{volts} \qquad \text{Gibbs equation} \\ &= -13.1\ \text{kcal/mol of DHA reduced} \end{aligned}$$

$$\begin{aligned} \Delta G^{\circ} &= -RT\ \ln K \\ \frac{-13.1\ \text{kcal}}{\text{mol}} &= -1.987 \times 10^{-3}\ \frac{\text{kcal}}{\text{mol }^{\circ}\text{K}} \times 310^{\circ}\text{K}\ \ln K \qquad \text{Gibbs equation} \\ \ln K &= 21.26 \\ K &= 1.7 \times 10^{9} \end{aligned}$$

$$\text{Log } K = \frac{(\Delta E^{\circ}) \times n}{RT/F}$$

$$= \frac{(0.284)\ (2)}{0.0615} = 9.23 \qquad \text{Nernst equation}$$

$$K = 1.7 \times 10^{9}$$

$$K = \frac{[GSSG]\ [AH_2]}{[GSH]^2\ [DHA]},\ \left.\begin{array}{l} GSH = 10^{-2}\ M \\ GSSG = 0.05 \times 10^{-2}\ M \end{array}\right\} \text{in rat lens}$$

$$1.7 \times 10^{9} = \frac{[0.05 \times 10^{-2}]\ [AH_2]}{[10^{-4}]\ [DHA]}$$

$$\frac{[AH_2]}{[DHA]} = \frac{1.7 \times 10^{9} \times 10^{-4}}{0.05 \times 10^{-2}} = 3.4 \times 10^{8}$$

In the rat lens, ascorbate is $\approx$ 50 mg/l = 2.8×10^{-4} M (ref. 13)

$$\frac{[AH_2]}{[DHA]} = 3.4 \times 10^{8}$$

$$\text{Approximate [DHA]} = \frac{2.8 \times 10^{-4}}{3.4 \times 10^{8}}\ \text{M} = 0.82 \times 10^{-12}\ \text{M}$$

$\Delta E^{\circ\prime}$ = Sum of the reduction potentials of both the half reactions
$\Delta G^{\circ\prime}$ = Gibbs energy of the reaction
n = Number of electrons involved in the redox reaction
F = Faraday, expressed in terms of kcal/mol °K volt
R = Gas constant in terms of kcal/mol °K
T = Absolute temperature in Kelvin
K = Equilibrium constant
In rat lens, [GSH] = 10^{-2} M, [GSSG] = 0.05×10^{-2} M
Ascorbate = 2.8×10^{-4} M
(For conventions, see ref. 21.)

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DISCUSSION OF THE PAPER

L. MACHLIN (*Hoffmann-La Roche Inc., Nutley, N.J.*): In your last slide, there was quite a substantial difference in the aqueous humor between the cortical and nuclear cataracts but no difference in the lens. I thought you said that it's the uptake in the aqueous humor that is the critical factor?

S. D. VARMA (*University of Maryland School of Medicine, Baltimore, Md.*): The lenses are not different because they are both cataracts taken by intracapsular extraction. When you estimate ascorbic acid you don't know what you're measuring because those lenses are porous. The aqueous humor estimations are more reliable. We hypothesize that the difference between cortical and nuclear cataract ascorbic acid is related to the transport of ascorbic acid from the blood to the aqueous humor. This needs confirmation. The number of cases is not that large, but to get aqueous humor is very difficult.

UNIDENTIFIED SPEAKER: Most of your data was with the rat. Nocturnal animals don't have high concentrations of ascorbate in the aqueous humor and might not need this kind of mechanism.

S. D. VARMA: We have said in a very early paper that the concentration of ascorbic acid is higher in the diurnal animals compared to the nocturnal animals. Also, the concentration of ascorbic acid is lower in the animals in utero than postnatally. So there could be some component involving their adaptation to the light and that's why there is apparently a higher concentration of ascorbic acid in diurnal animals.

H. SPRINCE (*Jefferson Medical College, Philadelphia, Pa.*): From your slide showing the riboflavin liberation of oxygen, may I infer that the high doses of riboflavin could generate the formation of cataracts?

S. D. VARMA: Possibly.

H. SPRINCE: Are people at risk with high doses of riboflavin?

S. D. VARMA: Well, any photosynthetizer at a high dose will be bad because it will initiate unwanted photochemical reactions.

A. TAYLOR (*Tufts University, Boston, Mass.*): You made a distinction between nuclear and cortical opacities in the Emory mice and I think that you showed animals on higher E diets have delayed onset of that cataract. We have shown in the same mice that dietary restriction delays the onset of cataracts.

About 15 years ago we carried out studies on the distribution of [^{14}C]ascorbic acid in guinea pigs. At that time we were very much surprised that the lens accumulated the radioactivity to a large extent. In addition we were surprised that the half-life of retention of lens ascorbic acid was very similar to the half-life we found in brain and in testes.

S. D. VARMA: It is very difficult to deplete the lens of ascorbic acid.